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VESTAR, INC.



April 16, 1992

Scientific Officer
Naval Medical Research and Development Command
Attention: CDR Robert Carter
Director, Research and Development
Ref: Contract N00014-92-C-0020
Bethesda, MD 20889-5044

Subject: Interim Report on Lyophilization of Liposome Encapsulated Hemoglobin

**General**

This report covers the research performed on contract N00014-92-C-0020 starting from January 92 through March 92. Research was completed in general conformance to the Phase I Work Plan described in the proposal Topic #N91-313 submitted for this SBIR.

Progress made on the ONR contract #N00014-90-C-0195 "Development and Technology Demonstration of Liposome Encapsulated Hemoglobin" has resulted in at least one alternate formulation candidate to the original NRL formulation of Liposome Encapsulated Hemoglobin (LEH). However, because the new candidate formulation has not progressed to the same level as the original, both were evaluated in parallel. The original NRL formulation is Distearoylphosphatidylcholine (DSPC), Cholesterol (CHOL), Dimyristoylphosphatidylglycerol (DMPG), and alpha-tocopherol ($\alpha$-T) in 10:9:1:0.4 molar ratio. The candidate Vestar formulation is a mole ratio of 7:10:3:0.4 of the same components. As has been discussed with NRL personnel, the primary reasons for the formulation change are size distribution and filterability.









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**Materials**

DSPC and DMPG were purchased from Avanti Polar Lipids. CHOL was sourced from EM Sciences. Recombinant Hemoglobin (RHb) was supplied by Somatogen. Sucrose came from EM Science. Lactose was provided by Mallinckrodt. Alpha-Tocopherol was provided by Spectrum. Trehalose was purchased from Sigma.

**Formulation**

All formulation work was done at Vestar. For the purposes of this SBIR, it was necessary to exchange the buffer solution of the Somatogen RHb from phosphate buffered saline to phosphate (5 mM) buffered disaccharide. This took the form of sucrose, lactose, or trehalose. The buffer exchange was achieved using commonly practiced dialysis procedures in refrigerated (+5° C) conditions.

**Cycle Development**

Four experimental cycles have been run. The Vestar lyophilization cycle for AmBisome was utilized as a starting point. This cycle starts at room temperature for product loading, ramps to a -45° C freezing point, holds at that temperature for a minimum of 6 hours, ramps to a 20° C primary drying set point, and ends with a 25° C secondary drying set point. These are idealized set points and actual cycle performance lags programmed ramp times somewhat because of dryer shelf mass and product loading. All work was done with a Vestar modified Virtis shelf-type lyophilizer capable of shelf stoppering of vials. A Honeywell programmable controller is used for cycle control. Data is automatically entered into Vestar's Data Acquisition system during the cycle. This permits a complete graphical representation of the cycle or a point by point data summary of the critical cycle parameters of shelf, product, and condenser temperatures together with chamber vacuum.

Test run #1 was essentially a "shakedown" run using three different formulations to evaluate the ruggedness of LEH to the AmBisome cycle when LEH is presented in a variety of forms with various excipient mixes. Fills of the 6 ml vials were at 2.5 m each. Formulations were: #1 - NRL formula with 30 mM phosphate, 7.3% saline, and 8.5% sucrose; bovine Hb was

used. #2 - NRL formulation with 30 mM phosphate, 7.3% saline, no sucrose, RHb. #3 - Vestar 7:10:3 with 5 mM phosphate, 9% sucrose (inside and outside the vesicles), RHb. The post-cycle examination indicated that the cakes all looked good, i.e. structurally sound with minimal to no appearance of collapse or meltback. Mean sizes also did not change significantly. Thus, the cycle posed a reasonable starting point.

Test run #2 was a comparative sucrose, lactose, trehalose test using the NRL formulation with any one of the disaccharides, 30 mM phosphate, and 7.3% saline with RHb, and the Vestar 7:10:3 (V-1) with any one of the disaccharides, 5 mM phosphate and RHb. Results were uniformly good with minimal structural effects. Sample volumes were 7.5 ml in a 10 ml vial giving the desired 2 cm fill height. The appearance of small structural effects at this fill height using a 20° C primary drying temperature were not unexpected.

Test run #3 was done with NRL in 9% sucrose, 30 mM phosphate, 7.3% saline, and V-1 in 9% sucrose, 5 mM phosphate; all other aspects the same as Test #2. The performance of sucrose in Test #2 was judged reasonably close to the other disaccharides with some possible benefit to the non-reducing behavior of sucrose. Large-scale use of sucrose in parenterals led us to proceed with it for all subsequent experiments. Test #3 utilized a +10° C primary drying temperature with no apparent signs of structural problems. Reconstitution was markedly superior with V-1 taking less than two minutes with initial dissolution very rapid. NRL formulation commonly took 30 minutes to reconstitute completely.

Test run #4 further explored the effect of lowering primary drying temperature. We have found in similar work that a specific product temperature profile during drying results in superior reconstitution performance. This had not been achieved in earlier runs. Thus, lowering the primary drying temperature to 0° C was tried. No evidence of structural problems was seen. Samples were the same as Test #3. Reconstitution was again superior with V-1. The reconstitution performance of the NRL formulation may in part be due to the presence of saline at 7.3%. This is in conformance to NRL methods, however a

separate test without saline seems warranted in view of the marked difference in reconstitution performance.

**Assays**

A. Encapsulation

An assay has been developed using a packed column which exhibits reasonable precision, accuracy, reproducibility, and ruggedness at exterior Hb concentrations of $\leq$ 50%. Above that value, some evidence of overloading of the column appears. Since lyophilized preparations are always less than 40% free Hb, they are within the appropriate range for the assay. Most preparations are < 5% free Hb pre-lyo. This assay was only recently completed, and results will be presented in the SBIR final report.

B. Size

Liposome size distributions were analyzed by Leeds and Northrup Microtrac, a laser light scattering machine. Although limited runs have been analyzed, the trend is clear that lyophilization has minimal effect on size distribution. In fact, mean size is usually seen to decrease slightly compared to pre-lyophilization. This is an interesting phenomenon, especially considering that electron micrograph (EM) studies have shown that the NRL size distribution visually is approximately 0.2-0.3 microns but is full of aggregates. These may disassociate temporarily because of lyophilization.

C. % Moisture

Cycles to-date have exhibited residual % moistures in the range of 0.5%, well within the acceptable 3% maximum characteristic of stable lyophilized products.

D. Methemoglobin

This assay is presently under way using the NRL method of octyl-6-glucopyranoside and chloroform. Preliminary results indicate minimal effect on met Hb formation.

E. $P_{50}$

Sample analysis is presently under way using the Hemox analyzer. Minimal changes have been seen to-date.

F. Lipids, Sterility, Pyrogen

There are as yet no results for these assays. These were planned for late in the program.

**Summary**

Progress to-date is encouraging. The AmBisome cycle works reasonably well for LEH with some modifications. Preliminary assay results indicate minimal effect of lyophilization on either formulation of LEH. Further work will center on additional cycle modifications in regards to ramping rates. All future runs will characterize the major factors of size, encapsulation, $P_{50}$, and met Hb, both pre- and post-lyophilization. The final report will present all pertinent analytical data and detailed lyophilization cycle parameters in tabular and graphical form as appropriate.

Sincerely,

Kevin R. Bracken
Senior Director, Process Development

Attachment: Form DD250

cc: Vestar
P. Schmidt
J. Short
G. Fujii
N. Sobieck
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U.S. Government
Administrative Contracting Officer - Santa Ana
Director Naval Research Laboratory
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| 9. PRIME CONTRACTOR CODE | 10. ADMINISTERED BY CODE |
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| Vestar, Inc.<br>650 Cliffside Drive<br>San Dimas, CA 91773 | DCMAO Santa Ana<br>34 Civic Center Plz.<br>P.O. Box C-12700<br>Santa Ana, CA 92712-2700 |
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